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Isatisine A, a Novel Alkaloid with an Unprecedented Skeleton from Leaves of *Isatis indigotica*

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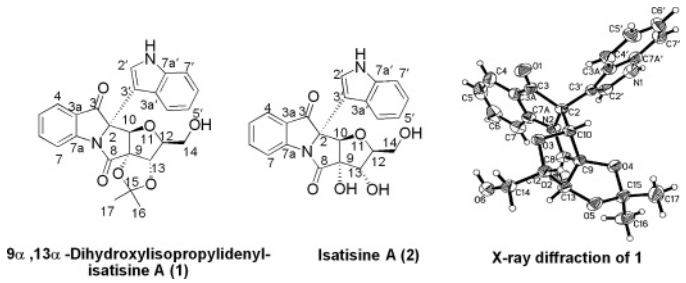
ABSTRACT



9α,13α-Dihydroxylisopropylidenylisatisine A (1), which was derived from isatisine A (2) and possessed an unprecedented fused pentacyclic skeleton, was isolated from the leaves of *Isatis indigotica* Fort. The structure and relative configuration were elucidated on the basis of extensive NMR analyses and finally determined by single-crystal X-ray diffraction. Compound 1 showed moderate anti-HIV-1 activity with EC_{50} = 37.8 μM and SI = 7.98.

Isatis indigotica Fort. (Cruciferae) is a biennial herbaceous plant species widely distributed and cultivated in China. The roots and leaves, respectively, named "Ban-Lan-Gen" and "Da-Qing-Ye" in Chinese, have been used as a traditional Chinese medicine for the treatment of viral diseases including influenza, viral pneumonia, mumps, and hepatitis for hundreds of years in China.[1] Diverse structures and significant biological activities of this plant have been attracting considerable interest. Chemical investigation of this plant has led to the isolation of indigotin, indirubin, epigotrin, 2-hydroxy-3-butenyl thiocyanate, 3-(2′-hydroxyphenyl)-4(3*H*)-quinazolinone, purin, isaindigotidione, organic acids, and many amino acids.[2−6] Recently an anti-influenza virus effect of indirubin has been documented.[7]

To find an active anti-HIV compound from this plant, the leaves of *I. indigotica* were investigated, and we reported

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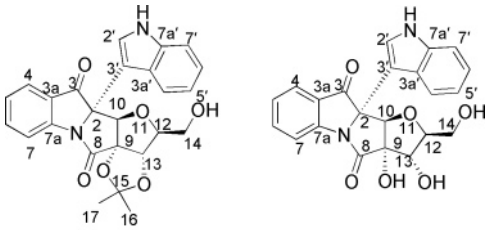
11 known compounds in our previous research.[8] During our study on this plant, a unique alkaloid was isolated. This paper deals with the isolation and structural elucidation of compound **1** through extensive spectroscopic analyses and single-crystal X-ray crystallography, as well as its anti-HIV-1 activity in vitro.

The leaves of *I. indigotica* were collected from Anhui province, China, in November 2004 and identified by professor Ya-qiu Zhou of Anhui College of Traditional Chinese Medicine (voucher No. 2004-11-5). The air-dried and powdered leaves (50 kg) were extracted three times with 80% EtOH for 2 h under reflux. The extract was concentrated under a vacuum to give a residue which was partitioned between petroleum ether, EtOAc, *n*-butanol, and water three times successively. After evaporation, the EtOAc fraction (120 g) was chromatographed on a silica gel column eluted with $CHCl_3$ and increasing amounts of MeOH (from 10:0 to 0:10, v/v) to give eight fractions A−H. Fraction C (6.5 g) was submitted to silica gel column chromatography (CC) with an eluent of petroleum ether/acetone (from 10:0 to 3:7) to afford fractions 1−6. Fraction 4 (1.2 g) was subjected to silica gel CC repeatedly eluting with petroleum ether/EtOAc (8:2) and further purified by Sephadex LH-20 (MeOH) to yield compound **1** (64 mg).



1 **2**

Compound **1**, $[\alpha]^{14}_{D}$ −283.15 (*c* 0.46, MeOH), was obtained as yellow needle crystals (MeOH/EtOH = 99:1, v/v).[9] The negative FAB MS gave a quasimolecular ion peak at 445 $[M - 1]^-$, in aggrement with the molecular formula of $C_{25}H_{22}N_2O_6$ revealed by negative HR-ESIMS, demonstrating 16 degrees of unsaturation in the molecule. The IR spectrum showed the absorptions for hydroxyl (3415 cm^{-1}), carbonyl (1717 cm^{-1}), and the aromatic ring (1603, 1470 cm^{-1}). The ^{13}C NMR spectrum exhibited 25 carbon resonances due to two methyls, one methylene, twelve methines, and ten quaternary carbons. The ^{13}C NMR and HSQC spectra allowed the assignments of all the protons to their bonding carbons. The ^1H NMR spectrum displayed eight aromatic protons at δ_H 8.02 (1H, br d, J = 8.1 Hz, H-7), 7.94 (1H, br d, J = 8.0 Hz, H-4′), 7.78 (1H, dd, J = 8.1, 7.5 Hz, H-6), 7.65 (1H, br d, J = 7.6 Hz, H-4), 7.38 (1H, br d, J = 8.2 Hz, H-7′), 7.33 (1H, dd, J = 7.6, 7.5 Hz, H-5), 7.16 (1H, dd, J = 7.9, 7.4 Hz, H-6′), and δ_H 7.09 (1H, dd, J = 7.4, 7.4 Hz, H-5′) (Table 1).

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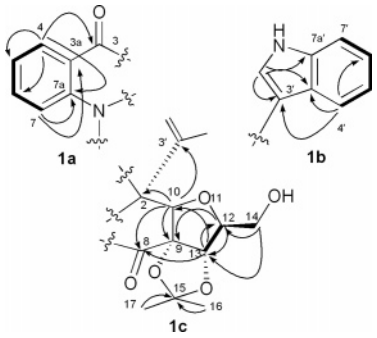
(9) Compound **1**: mp 209−210 °C; UV (MeOH) λ max (log ϵ) 217 (4.66), 240 (4.41), 258 (4.22) nm; IR (KBr) ν_{max} 3415, 2936, 1717, 1603, 1513, 1470, 1374, 1112, 1090, 748 cm^{-1}, NMR data found in Tabel 1; negative FAB MS *m*/*z* (rel. int.) 445 (100, $[M - H]^-$), 327 (15), 245 (30); the negative HR-ESIMS found 445.1410, calcd for $C_{25}H_{21}N_2O_6$ 445.1399.

Table 1. ^1H and ^{13}C NMR Data of Compound **1** (in CD_3OD)[a]

no.	δ_H (mult., J, Hz)	δ_C
2		76.3, s
3		195.7, s
3a		127.3, s
4	7.65, br d, 7.6	126.2, d
5	7.33, dd, 7.6, 7.5	127.0, d
6	7.78, dd, 8.1 7.5	137.9, d
7	8.02, br d, 8.1	117.4, d
7a		151.2, s
8		171.4, s
9		99.4, s
10	4.91, s	85.9, d
12	4.17, m	87.1, d
13	4.81, d, 3.3	87.8, d
14a	3.51, dd, 12.0, 4.2	62.5, t
14b	3.44, dd, 12.0, 4.4	
15		119.4, s
16	1.51, s	26.3, q
17	1.38, s	27.3, q
2′	7.26, s	124.3, d
3′		111.0, s
3a′		125.7, s
4′	7.94, br d, 8.0	121.2, d
5′	7.09, dd, 7.4, 7.4	120.7, d
6′	7.16, dd, 7.9, 7.4	123.3, d
7′	7.38, br d, 8.2	112.9, d
7a′		139.1, s

[a] ^1H NMR recorded at 500 MHz; ^{13}C NMR recorded at 125 MHz.

The partial structure of an ortho-substituted aromatic ring **1a** (Figure 1) was established by $^1H-^1H$ COSY (H-4/H-5, H-5/H-6, and H-6/H-7) and HMBC (H-4/C-3). Similarly, the detected correlations in $^1H-^1H$ COSY (H-4′/H-5′, H-5′/H-6′, and H-6′/H-7′) and HMBC (δ_H 7.26 correlated with C-3′, C-3a′, and C-7a′) established the fragment **1b** (Figure 1). Besides the partial structures of **1a** and **1b**, an isopropylidenyl unit was observed in the ^1H NMR (δ_H 1.38 and 1.51) and ^{13}C NMR [δ 119.4 (s), 26.3 (q), and 27.3 (q)] spectra. The presence of an isopropylidenyl unit in the molecule of compound **1** can also be supported by the HMBC spectrum in which the correlations between H-16 (δ_H 1.51, s, 3H), H-17 (δ_H 1.38, s, 3H), and C-15 were



1a **1b** **1c**

Figure 1. Fragment structures and key COSY (−) and HMBC (→) corrections of compound **1**.

displayed. The other correlations in the HMBC spectrum of compound **1** can be found as follows: H-10 (δ_H 4.91, s) with C-2, C-8, C-9, and C-3′; H-12 (δ_H 4.17, m) with C-9 and C-13; H-13 (δ_H 4.81, d, J = 3.3 Hz) with C-8, C-10, C-12, and C-14; H-14a (δ_H 3.51, dd, J = 12.0, 4.2 Hz) and H-14b (δ_H 3.44, dd, J = 12.0, 4.4 Hz) with C-12 and C-13. The above-mentioned HMBC correlation evidence, combined with the cross-peaks of H-14/H-12 and H-12/H-13 in the ^{1}H−^{1}H COSY, led to the establishment of fragment **1c** (Figure 1).

Unfortunately, the 1D and 2D NMR spectra did not provide enough information to establish the linkages of C-2, C-3, C-3′, and N-1. Thus, a single crystal of compound **1** was obtained from MeOH/EtOH (99:1, v/v), and X-ray crystallographic analysis was conducted (Figure 2),[10] which

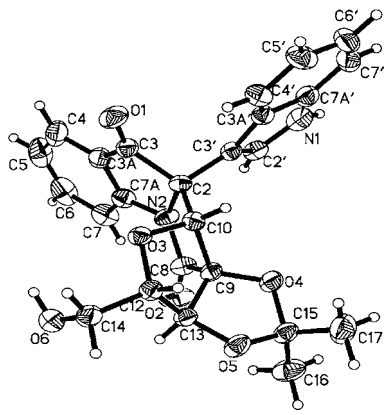


Figure 2. X-ray structure of **1** showing relative configuration.

clarified the uncertain structure and the relative stereochemistry of compound **1** as proposed and named as 9α,13α-dihydroxylisopropylidenylisatisine A. According to the IUPAC nomenclature rule and based on the chiral carbon atom with the lowest locant, the absolute stereochemistry of C-2, 9, 10, 12, and 13 was deduced as *R**, *S**, *R**, *S**, and *S**, respectively.

As far as we know, this is the first report of an alkaloid from *I. indigotica* possessing such a unique skeleton. Natural compounds containing the isopropylidenyl group have often been reported;[11−15] however, we did not know whether compound **1** was a natural product or an artifact from the experimental procedure considering the acetone as a solvent used in our purification process. To confirm the origin of compound **1**, the EtOAc fraction (1.8 g) was chromatographed on a silica gel column (petroleum ether/EtOAc, 9:1 to 1:9, v/v) to give seven fractions (1−7). TLC and HPLC were used to detect this component by comparison with the authentic sample of compound **1** (for detailed experiments, see Supporting Information), and compound **1** could not be detected in fractions 1−7, which suggested that compound **1** might be an artifact from the isolation procedure as reported.[16] Furthermore, compound **1** was acid-hydrolyzed to afford isatisine A (**2**) ($C_{22}H_{18}N_2O_6$).[17] TLC and HPLC analyses of hydrolysates demonstrated that isatisine A (**2**) could be detected in fraction 5 (for detailed experiments, see Supporting Information). Therefore, we were inclined to consider that compound **1** was an artifact and that isatisine A (**2**) was a genuine natural product in this plant.

Although compound **1** has been proved to be an acetonide of compound **2**, isatisine A (**2**) is a novel alkaloid possessing an unprecedented fused-pentacyclic skeleton (fragment C-9 to C-13) which cannot be well explained from the biogenetic view. Compound **1** was tested for cytotoxicity activity against C8166 cells (CC_{50}) using the MTT method as reported,[18] and anti-HIV-1 activity was evaluated by the inhibition assay for cytopathic effects of HIV-1(EC_{50}).[19] The compound exerted cytotoxicity against C8166 with CC_{50} = 302 μM and showed anti-HIV-1_{IIIB} activity with EC_{50} = 37.8 μM and SI (selectivity index) = 7.98.

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Supporting Information Available: General experiment and spectra (UV, IR, MS, NMR) of compound **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(10) Crystallographic data of compound **1**: $C_{25}H_{22}N_2O_6$, M = 446.51, orthorhombic, space group $P222$, a = 9.8873 (16) Å, b = 11.8185 (19) Å, c = 20.1890 (3) Å, α = 90.000, β = 90.000, γ = 90.000, V = 2359.2 (7) Å^3, Z = 4. Crystal dimensions 0.09 × 0.21 × 0.38 mm^3 were used for measurements on a MAC DIP-2030K diffractometer with a graphite monochromator (ω scan, 2θ max = 56.74), Mo Kα radiation. The total number of independent reflections measured was 15 384, of which 5539 were observed ($|F|^2 \geq 2\sigma|F|^2$). Final indices: R_f = 0.0579, wR2 = 0.1220 ($w = 1/\sigma|F|^2$), S = 1.004. The crystal structure (**1**) was solved by the direct method SHELXS-97, expanded using geometrical calculations and difference Fourier techniques, and refined by least-squares calculations. Crystallographic data for the structure of compound **1** have been deposited in the Cambridge Crystallographic Date Centre (deposition number: CCDC 652447). Copies of these data can be obtained free of charge via http://www.ccdc.cam.ac.uk/deposit (or from Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, U.K., Facsimile: (44) 01223 336033, e-mail: deposit@ccdc.cam.ac.uk).

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(17) Compound **2**: $C_{22}H_{18}N_2O_6$, ^{1}H NMR (500 MHz, CD_3OD) δ = 7.99 (1H, d, J = 8.5 Hz, H-7), 7.93 (1H, d, J = 8.0 Hz, H-4′), 7.77 (1H, dd, J = 7.5, 7.5 Hz, H-6), 7.63 (1H, d, J = 7.5 Hz, H-4), 7.33 (1H, d, J = 8.0 Hz, H-7′), 7.32 (1H, dd, J = 7.5, 7.5 Hz, H-5), 7.28 (1H, s, H-2′), 7.12 (1H, dd, J = 8.0, 7.0 Hz, H-6′), 7.05 (1H, dd, J = 7.5, 7.5 Hz, H-5′), 4.63 (1H, s, H-10), 4.05 (1H, d, J = 4.0Hz, H-13), 3.83 (1H, m, H-12), 3.38 (1H, dd, J = 11.5, 4.5 Hz, H-14a), 3.33 (1H, dd, J = 11.5, 4.5 Hz, H-14b). The negative FAB MS: m/z (%) = 405 ([M − H]$^-$, 10), 353 (10), 339 (100), 325 (90), 311 (40).

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